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Human fear acquisition deficits in relation to genetic variants of the corticotropin-releasing hormone receptor 1 and the serotonin transporter – revisited

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We recently showed that a genetic polymorphism (rs878886) in the human corticotropin-releasing hormone receptor 1 (*CRHR1*) is associated with reduced fear-conditioned responses to a threat cue. This is a potentially important finding considering that the failure to acquire fear contingencies can leave an individual in a maladaptive state of more generalized anxiety. Consistent with that idea, the *CRHR1*-dependent fear acquisition deficit translated into heightened contextual anxiety when taking genetic variability within the serotonin transporter long polymorphic region (*5-HTTLPR*) into account. To replicate our previous findings, we conducted a replication study in 224 healthy medication-free human subjects using the exact same cue and context virtual reality fear-conditioning procedure as in study by Heitland *et al.* (2013). In the replication study, consistent with the original findings, *CRHR1* rs878886 G-allele carriers showed reduced acquisition of cue-specific fear-conditioned responses compared with C/C homozygotes. Also, in this larger sample the cue acquisition deficit of G-allele carriers translated into heightened contextual anxiety, even independent of *5-HTT* gene variation. In contrast to our earlier findings, there was an additional interaction effect of *CRHR1* rs878886 and the triallelic *5-HTTLPR*/rs25531 variant on cued fear acquisition. In summary, this study replicated the initially reported association of the *CRHR1* rs878886 G-allele with cued fear acquisition deficits, albeit with a different pattern of results regarding the interaction with *5-HTT* variation. This further supports the notion that the human corticotropin-releasing hormone plays a role in the acquisition of fears.

Keywords: Corticotropin-releasing factor, corticotropin-releasing hormone, CRHR1, fear conditioning, fear-potentiated startle, replication study, rs878886

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When it comes to the issue of replicability of scientific findings, a recent commentary (Lakens *et al.* 2012) stated: ‘One study is no study’. While it is certainly an exaggeration when taken literally, it illustrates a very valid and serious concern in human behavioral genetic research: very low replication rates. In order to address the validity of one of the findings from our own laboratory, we aimed to replicate results on the role of a single-nucleotide polymorphism (SNP) within the human corticotropin-releasing hormone receptor 1 (*CRHR1*, rs878886) gene in fear acquisition and respective deficits (Heitland *et al.* 2013) and investigated how *CRHR1* (rs878886) interacts with *5-HTTLPR*.

Fear acquisition is a clinically relevant process that is often assessed in humans by means of fear conditioning, which serves as the preferred laboratory model for the study of anxiety disorders (Duits *et al.* 2015). In fear conditioning, an initially neutral conditioned stimulus (CS, e.g. a light) is repeatedly paired with an inherently aversive event (unconditioned stimulus, UCS, e.g. an electrical shock). During the course of this acquisition process, conditioned fear responses develop toward the threat cue. At the same time, absence of the threat cue may come to signal periods of safety (Pavlov 1927). However, if this CS-UCS contingency is not acquired, threat remains unpredictable. This can result in chronic states of maladaptive anxiety in the context in which the CS is presented. Increased sustained fear as a result of the failure to acquire cued fear has been demonstrated both experimentally (Baas & Heitland 2014; Baas *et al.* 2008; Grillon 2002) and clinically, e.g. in panic disorder patients (Wolpe & Rowan 1988) (We use the term ‘increased sustained fear’ as defined by Davis *et al.* (2010) throughout the manuscript to describe the tonic, more long-lasting and contextual fear responses.). This study aims to replicate our previous findings on the impact of genetic variation in the *CRHR1* gene (rs878886), in interaction with 5-HTTLPR, on individual differences in fear acquisition (Heitland *et al.* 2013).

The corticotropin-releasing factor [CRF, also referred to as corticotropin-releasing hormone (CRH)] is one of the key neurotransmitters involved in the acquisition and regulation of fear, anxiety and stress (Davis 2006; Fox & Lowry

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2013). CRF exerts its effects via regulating activity in the hypothalamic-pituitary-adrenal axis (Hauger *et al.* 2006) and in extra-hypothalamic regions. These are, among others, the extended amygdala (Griebel & Holsboer 2012), including the bed nucleus of the stria terminalis (BNST) (Davis *et al.* 2010; Lee & Davis 1997) and the medial prefrontal cortex (Lowry & Moore 2006). Accordingly, CRF has been suggested as a crucial factor in the pathogenesis of stress-related psychopathologies such as anxiety (Hauger *et al.* 2006). This notion is supported in humans by observed CRH dysregulation in mood and anxiety disorders as demonstrated by altered CRH levels (Baker *et al.* 1999; Sautter *et al.* 2003), altered CRH_1 receptor expression (Bissette *et al.* 2003; Raadsheer *et al.* 1994) or CRH genetics (Binder & Nemeroff 2010; Ishitobi *et al.* 2012; Keck *et al.* 2008; Weber *et al.* 2015). As a potential mechanism behind the latter associations, the role of CRH in fear acquisition deficits has often been discussed (Bijlsma *et al.* 2011; Liang & Lee 1988; Roozendaal *et al.* 2002). Data from rodent studies support this hypothesis. For example, repeated local infusion of CRH into the basolateral part of the amygdala facilitates the acquisition of cue conditioned fear (Bijlsma *et al.* 2011). Consistent with these findings, CRH levels in the amygdala increase during fear acquisition in rodents (Roozendaal *et al.* 2002). Moreover, pharmacological blockade of the CRH_1 receptor prevents the acquisition and expression of context conditioned fear (Risbrough *et al.* 2009; Roozendaal *et al.* 2008). Recent studies suggest that CRH exerts its effects on cued/phasic fear via the central nucleus of the amygdala (CeA), whereas contextual/sustained fear is mediated mainly by the BNST (see Davis *et al.* 2010) for an overview of both models).

The serotonin system has often been linked to fear and anxiety, and a functional variant in the promoter region of the serotonin (5-HT) transporter gene referred to as *5-HTTLPR* has been associated with stronger fear expression (Klumpers *et al.* 2014; Munafò *et al.* 2008), also in the context of (conditioned) threat cues (Klumpers *et al.* 2011; Lonsdorf *et al.* 2009). *5-HTT*LPR seems to affect fear expression, but it may also impact acquisition (Lonsdorf *et al.* 2009). However, it interacts with CRF with regard to the regulation of anxiety-like responses (Lukkes *et al.* 2009) and the acquisition of context conditioning (Bijlsma *et al.* 2015; Heitland *et al.* 2013).

Given the theoretical potential of CRF_1 antagonists and the encouraging animal data, it might seem surprising that there still is a great paucity of human translational studies. This is most likely due to the fact that safe and approved pharmacological tools are lacking as of yet. To our knowledge, only two studies have investigated anxiolytic effects of CRHR1 antagonists in healthy humans (Bailey *et al.* 2011; Grillon *et al.* 2015). In the study by Bailey *et al.* (2011), acute anxiolytic effects of the CRH_1 receptor antagonist R317573 were demonstrated in an experimental CO_2 anxiety provocation model. In the study by Grillon *et al.* (2015), administration of the CRF_1 antagonist GSK561679 led to increased fear responses toward predictable threat. Despite these promising proof-of-concept studies, CRH1 antagonist research is still hampered by the fact that development of these agents is still in early preclinical phases.

A valid alternative to the administration of pharmacological agents is the study of innate variability within the target neurotransmitter system. As a human proxy for knockout studies in animals, this can be done by investigating genetic polymorphisms that have been associated with the behavior of interest (fear/anxiety) and are located within regulatory regions of the gene. We used this approach in our original study (Heitland *et al.* 2013), where we genotyped a SNP within the human *CRHR1* gene (rs878886) that had been linked to panic disorder (Keck *et al.* 2008) and subjected a sample of 150 healthy human participants to a well-established fear-conditioning paradigm. Fear potentiation of the eyeblink startle reflex (fear-potentiated startle, FPS) was used to index basic defensive state physiology in response to both cues and context. In accordance with the original hypothesis, *CRHR1* variation (rs878886) was associated with the acquisition of fear-conditioned responses. The serotonin (5-HT) transporter gene referred to as *5-HTT*LPR/rs25531 did not affect cue conditioning, but it was associated with heightened contextual anxiety in *CRHR1* G-allele carriers that did not acquire cued fear (Heitland *et al.* 2013).

Importantly, another recent study has shown indirect evidence for functional significance of rs878886 regarding gene expression and fear processing in humans (Weber *et al.* 2015). Using a combination of genetic analysis within *CRHR1*, personality assessments, behavioral data, physiological experiments and functional magnetic resonance imaging (fMRI), the authors demonstrated that the minor allele of another polymorphism in *CRHR1*, rs17689918, was associated with a phenotype characterized by heightened fear sensitization and increased anxious apprehension. Particularly interesting is that the minor risk allele of rs17689918 is associated with decreased CRHR1 mRNA expression in forebrains and amygdalae of human post-mortem brains (Weber *et al.* 2015), demonstrating a functional impact of genetic variability within *CRHR1*. Rs878886 is located in the 3′ UTR of *CRHR1*, and importantly, rs878886 and rs17689918 are in perfect linkage disequilibrium ($D' = 1$, $r^2 = 1$; 1000 genomes dataset, phase 1, version 3, March 2012). Hence, impact of rs878886 on gene expression has been shown indirectly, giving additional rational for studying genetic variability in *CRHR1* in the acquisition of conditioned fear. Importantly, the direction of the findings of Weber *et al.* (2015) is consistent with the reported rs878886 findings (Heitland *et al.* 2013; Keck *et al.* 2008).

As of yet, our own experimental findings regarding rs878886 and fear acquisition (Heitland *et al.* 2013) remain unreplicated, which greatly limits their potential implications for future research. Therefore, we here conducted a direct replication study. A total of 224 healthy human subjects completed the exact same experiment as in the original report (Heitland *et al.* 2013) and genetic variability in *CRHR1* (rs878886) and *5-HTT* (5-*HTT*LPR/rs25531) was determined.

Methods

Replication study

As this study is an exact replication study of what we earlier reported on, all study procedures are identical to (Heitland *et al.* 2013) unless

stated otherwise. In the following, a brief description of the study procedures is given nonetheless. For further details, the reader is referred to the original report (Heitland *et al.* 2013).

Ethics statement

All study procedures have been approved by the ethical institutional review board of the University Medical Centre Utrecht, and all subjects gave written informed consent. Furthermore, all study procedures have been conducted according to the principles expressed in the Declaration of Helsinki.

Subjects

A total of 224 subjects (156 females, 68 males; mean age = 22.19 years, SD = 2.78 years) were recruited via advertisements at Utrecht University. All participants were Caucasians of Western European descent and reported to be free of any current or previous psychiatric or neurological disorder, drug or alcohol dependence, current psychoactive medication, hearing problems and color blindness. Participants received 10 euros/h for their participation in the experiment. A total of 19 subjects were excluded from the final sample due to incomplete recordings of startle data or artifacts yielding unreliable startle measurements ($n = 17$), insufficient quality of isolated DNA ($n = 1$) or both ($n = 1$). The final sample therefore comprised 205 subjects between 18.2 and 33.3 years of age (141 females, 64 males; mean age = 22.1 years, $SD = 2.64$ years). Data of the current sample pertaining to fear extinction will be reported elsewhere (manuscript in preparation).

Experimental paradigm

All subjects completed the well-established FPS conditioning paradigm in a virtual reality environment as used and described in Heitland *et al.* (2013) to assess fear-conditioned responding to both a threat cue and a threat context. In this paradigm, subjects were presented with two virtual environments. These were an apartment in a high rise in a downtown area and a house in a suburban area (Baas *et al.* 2008). For each subject, one of the contexts was assigned as the threat context where shocks were administered (CXT+), whereas the other represented the safe context without shock reinforcement (CXT−). Assignment of the threat context and order of visits to the contexts was counterbalanced across subjects. An increase in background illumination (light on) with 8 seconds duration signaled when shocks could be administered in the threat context. Each block contained visits to both contexts, and a total of four lights on presentations in each context. Light on presentations in the safe context were never followed by shock and originally implemented to assess generalization of fear. As this phenomenon was not the focus of this study, data from this condition will be omitted for sake of brevity. Pictures from both contexts during light off and light on can be found elsewhere (Baas *et al.* 2008). Subjects were presented with the virtual environments in blocks lasting 5 min and 25 seconds during which both contexts were visited. The beginning of each block and transitions between contexts comprised transits through a virtual metro station during which startle probes were presented to maintain startle habituation (Baas 2013; Baas *et al.* 2008; Baas & Heitland 2014; Heitland *et al.* 2012, 2013).

The experiment was divided into three phases (see Fig. 1 for an illustration). In the first phase, six uninstructed acquisition blocks were presented to assess the development of uninstructed-conditioned responding and contingency awareness (uninstructed acquisition). During this phase, training blocks with a relatively high reinforcement rate of 75% to facilitate acquisition were alternated with testing blocks. Relatively low reinforcement rates (37.5% on average) during these test blocks and the transition to the next context after reinforcement prevented selective contamination of the assessment of physiological responding in the threat context due to shock sensitization (Baas 2013; Baas *et al.* 2008; Baas & Heitland 2014; Heitland *et al.* 2012, 2013). Therefore, only startle data from test blocks (blocks 2, 5, 6 and 8–11) are reported. The reinforcement rate in block 2 was always 50%, whereas block 5 and 6 had 25%/50% or 50%/25% reinforcement rate (order was counterbalanced across subjects). The uninstructed acquisition phase was followed by explicit verbal and written instructions about the contingency between threat context, threat cue and shock reinforcements to ensure contingency learning in all participants. These instructions were followed by one training block to reinforce the instructions and four testing blocks to assess instructed fear expression, the second phase of the experiment. Finally, subjects underwent an extinction phase after the fear expression phase (data will be reported elsewhere, manuscript in presentation).

Throughout the experiment, startle probes were presented during three out of four light on presentations in both contexts. In addition, three startles probes were presented in absence of the light cue in each context. These are further referred to as the light off condition. As a result, each block contained three startles measurements per condition (light on/CXT+; light off/CXT+; light off/CXT−; light on/CXT−).

Shock administration and workup

Electrical shocks were administered with a constant current generator (Digitimer DS7A, Digitimer Ltd., Letchworth Garden City, UK) via tin cup electrodes located approximately over the medial nerve on the inner left wrist. Before the experiment started, subjects completed a shock workup to determine individual shock intensities as described in previous publications (Baas & Heitland 2014; Heitland *et al.* 2012, 2013; Klumpers *et al.* 2010b, 2011). Intensities were adjusted per subject so that they corresponded to a level of 4 out of 5, representing 'quite annoying/painful'.

Startle probe presentation, data recording and processing

Recording and amplification of the eyeblink startle reflex was performed via electromyography of the right *orbicularis oculi* muscle using a Biosemi ActiveTwo system (BioSemi Instrumentation, Amsterdam, The Netherlands). Startle probes comprised 50 ms, 105 dB white noise bursts with instantaneous rise time and were delivered through headphones (Sennheiser Electronic HD202, Wennebostel, Germany). Processing of startle data was performed using Brain Vision Analyzer software (Brain Products, Gilching, Germany) according to published guidelines (Blumenthal *et al.* 2005) and previous studies (Baas & Heitland 2014; Heitland *et al.* 2012, 2013; Klumpers *et al.* 2010a). After segmentation of trials, artifacts were rejected and null responses identified as described previously (see Klumpers *et al.* 2010b) for procedural details and criteria). Participants were only included in the final analysis if at least one artifact-free startle trial for each condition and each block was recorded. Startle data were Z-transformed per subject based on individual trial amplitudes from all startles recorded during the experiment to remove between-subjects variance in baseline startle amplitude. All statistical analyses involving startle data were conducted on Z-scores.

Startle electrode placement

One EMG electrode was placed below the lower eyelid in line with the pupil in forward gaze, the second electrode was placed ~1 cm lateral to the first electrode. Of note, electrode placement of this second lateral electrode was slightly different in $N = 133$ out of the $N = 224$ subjects. In these participants, the second electrode was placed 2 cm lateral to the first one, resulting in overall lower startle amplitudes in these subjects ($M = 54.60$, $SD = 42.35$) when compared with the subjects in which this electrode was placed 1 cm lateral to the one below the pupil ($M = 91.53$, $SD = 61.09$). However, all startle data were Z-transformed, which accounted for potential confounding effects of these differences with regard to comparison of genetic groups. To preclude any unwanted variance and confounding effects of this in the genetic analyses, we included electrode placement as a covariate in all analyses of variance (ANOVA's) involving startle data. Importantly, all statistical test outcomes (significant or non-significant) reported in the following were identical with and without inclusion of this covariate.

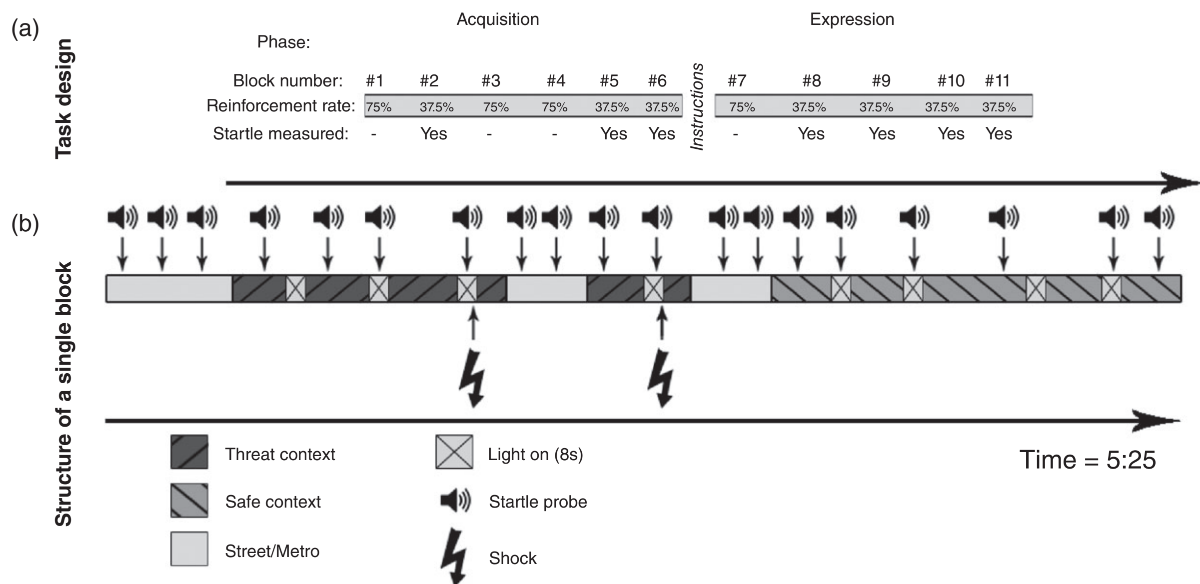

Figure 1: Illustration of the virtual reality fear-conditioning paradigm used in this study. (a) Design of the experimental task. (b) Overview of the movie composition from a single testing acquisition block. Note, that this is the exact same paradigm as described and used in (Heitland *et al.* 2013).

Subjective measures

Prior to the experiment, subjects filled out Dutch translations of the Spielberger's Trait Anxiety Inventory (Spielberger *et al.* 1970; Van der Ploeg 1980) and the neuroticism subscale of the NEO-PI-R questionnaire (Costa & McCrae 1992). In addition, subjects rated their subjective fearfulness between blocks of the virtual reality fear-conditioning paradigm. This was done using a visual analog scale displayed on the computer screen together with screenshots from the pre-recorded videos representative for each condition. See (Baas *et al.* 2008) for examples of screenshots. The question was 'How fearful do you feel in this situation?' with the anchors: 'Not at all fearful of shock' (0) and 'Very fearful of shock' (100). Two screenshots per condition were presented after each block, and an average rating was computed per condition and block. Further analysis of the data was congruent to our approach of the startle data, but data were not Z-transformed as the theoretical range of the scores was the same for every subject. In addition to these fearfulness ratings, shock contingency awareness was assessed by forced choice ratings of shock expectancy between blocks as described earlier (Baas *et al.* 2008).

Genotyping

DNA was harvested by collecting buccal swaps frozen immediately at −40°C for later genotyping. Genomic DNA was extracted and purified using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany).

CRHR1 rs878886 was genotyped using Taqman SNP Genotyping assays (ASSAY ID's: C___7450783_10; Applied Biosystems, Foster City, CA). Subjects were classified through endpoint analysis performed on an ABI Prism 7000 (Applied Biosystems) as either C/C homozygotes, C/G heterozygotes or G/G homozygotes. All *CRHR1* samples were genotype in duplicate, with no deviations observed. To achieve sufficient statistical power, we grouped C/G heterozygotes and G/G homozygotes as G-allele carriers. All genetic analyses regarding *CRHR1* rs878886 thus compared G-allele carriers with C/C homozygotes. *CRHR1* rs878886 genotyping was performed in duplicate for all the samples without deviations. *5-HTTLPR*/rs25531 genotyping was performed using established protocols (Wendland *et al.* 2006). Polymerase chain reaction with subsequent gel electrophoresis was performed, which visualized (per subject) either two short 486 bp DNA fragments (S/S), one short and one long (529 bp) fragment (S/L) or two copies of the long fragment (L/L). In addition to the biallelic genotyping procedures in the original study, the functional SNP rs25531, representing an A to G nucleotide substitution within the L allele of the 5-HTTLPR, was determined using a restriction endonuclease digest and gel electrophoresis (see Wendland *et al.* 2006 for details). Resulting haplotypes were thus either L_A or L_G. The L_A allele has been associated with high 5-HTT expression and activity, whereas the less frequent L_G allele is functionally very similar to the low expression/function S allele (Hu *et al.* 2006; Nakamura *et al.* 2000). To ascertain sufficient statistical power, we pooled triallelic *5-HTTLPR*/rs25531 genotypes into a high 5-HTT functioning (L_A/L_A) and low 5-HTT functioning (S/S, S/L_A, S/L_G, L_A/L_G and L_G/L_G) group as commonly done with the triallelic 5-HTTLPR (Gloster *et al.* 2015; Klumpers *et al.* 2014; Praschak-Rieder *et al.* 2007). *5-HTTLPR*/rs25531 genotyping was performed in a single run. Genotype frequencies and statistics such as total *N* per dataset, minor allele frequency, Hardy–Weinberg equilibrium, *N*'s per genotype, percentage of genotype are presented in Table 1. The genotype error rate was 0.009%, meaning 2 out of 222 subjects could not be genotyped for both polymorphisms, most likely due to insufficient quality of isolated DNA. (Of note, one of these two subjects also had incomplete startle recordings.)

Statistical analyses

For consistency, all statistical procedures used here were identical to our initial approach (Heitland *et al.* 2013). Cued fear was defined as potentiation to the threat cue within the threat context (cue FPS: light on/CXT+ vs. light off/CXT+). Contextual anxiety was defined as potentiation to the threat context in absence of the light cue (context FPS: light off/CXT+ vs. light off/CXT−). For clarity of presentation and coherence with our initial study, planned comparisons rather than full-factorial designs are reported. Note, however, that statistical outcomes reported in the following (significant vs. non-significant) are identical when using full-factorial designs and planned comparisons. For both cue FPS and context FPS, repeated-measures ANOVA's were conducted per phase (uninstructed acquisition and fear expression) using the contrasts stated above. *CRHR1* rs878886 genotype and *5-HTTLPR*/rs25531 genotype were included as between

Table 1: Frequencies and statistics (Hardy-Weinberg equilibrium, linkage equilibrium and gender distribution) of the genetic polymorphisms under study are shown

Polymorphism	Minor allele frequency	Hardy–Weinberg equilibrium	N's						Genotype (%)						Females (%)						*P* (gender × genotype)
CRHR1 (rs878886)	0.27	0.37	C/C	C/G	G/G				C/C	C/G	G/G				C/C	C/G	G/G				0.46
			108	85	12				53	42	6				69	66	83				
5-HTTLPR (bi-allelic)	0.41	0.25	S/S	S/L	L/L				S/S	S/L	L/L				S/S	S/L	L/L				0.51
			30	107	68				15	52	32				60	71	69				
5-HTTLPR/ rs25531 (tri-allelic)	—	—	S/S	S/L_G	S/L_A	L_G/L_G	L_A/L_G	L_A/L_A	S/S	S/L_G	S/L_A	L_G/L_G	L_A/L_G	L_A/L_A	S/S	S/L_G	S/L_A	L_G/L_G	L_A/L_G	L_A/L_A	0.50
			30	12	94	2	15	51	15	6	46	1	7	25	60	67	71	50	87	65	
5-HTTLPR expression profile	—	—	Low	High					Low	High					Low	High					0.36
			154	51					75	25					70	65					

The corresponding *CRHR1* [rs878886] × *5-HTTLPR* linkage equilibrium *P*-value = 0.15. For the triallelic *5-HTTLPR*/rs25531, high 5-HTT functioning was defined as: L_A/L_A carriers. Low 5-HTT functioning was defined as: S/S, S/L_G, S/L_A, L_A/L_G and L_G/L_G carriers.

Table 2: Frequencies, N per cell, age, STAI-T, NEO-N, shock intensity, baseline startle amplitude, percentage of null-responses per experimental phase and contingency awareness data are displayed for all possible genotype combinations, and for the whole sample

	5-HTTLPR	Descriptives		Age		STAI-trait		NEO-N		Shock intensity (mA)		Basline startle magnitude (μV)		Percentage of null responses (acquisition)		Percentage of null responses (expression)			
CRHR1 rs878886	Triallelic expression	*N's*	Females (%)	*M*	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*	*M*	SD	M	SD	Percentage of cue aware %	Percentage of context aware %
C/C	Low	80	68.6	22.2	2.8	37.0	8.4	30.4	8.6	1.5	0.9	62.5	50.0	3.1	4.3	6.4	8.4	38.8	86.1
	High	28	71.4	22.3	2.5	35.9	7.3	31.0	6.7	1.4	0.7	77.2	42.5	3.7	6.1	8.7	11.4	42.9	89.3
G carrier	Low	74	71.6	21.8	2.4	36.7	8.0	31.5	7.7	1.8	2.1	74.1	61.7	2.9	4.5	6.3	8.2	36.5	89.2
	High	23	56.5	22.6	3.2	34.5	8.4	29.5	7.7	2.3	2.7	77.2	54.5	2.1	3.6	5.5	9.0	39.1	95.7
Whole sample		205	68.8	22.1	2.6	36.5	8.1	30.8	7.9	1.7	1.7	70.4	54.2	3.0	4.6	6.6	8.8	38.5	88.7

For the triallelic *5-HTTLPR*/rs25531, high 5-HTT functioning was defined as: L_A/L_A carriers. Low 5-HTT functioning: S/S, S/L_G, S/L_A, L_A/L_G and L_G/L_G carriers. Cue and context awareness was determined at the end of the acquisition phase, before insturctions were given.
NEO-N, Revised NEO Personality Inventory neuroticism subscale; STAI-trait: Spielberger's trait anxiety inventory.

subjects' factors with two levels per genotype. In addition, the *CRHR1* rs878886 × *5-HTTLPR*/rs25531 interaction was entered as a between-groups factor. There were no statistically significant interaction effects between the genetic factors under study and the factor block on any of the analyses performed. Details on statistics and data plots that include the factor block are therefore omitted from the main article and can be found in Figure S1, Supporting Information. Gender and age were added as covariates for all statistical comparisons that involved the genetic polymorphisms under study as commonly done in behavioral genetic research. Of note, all statistical test outcomes (significant or non-significant) reported in the following were identical with and without inclusion of these covariates.

Results

Descriptive statistics

Genotype and gender distribution, mean shock intensities, baseline startle amplitudes, trait anxiety scores and neuroticism scores as well as null response rates and contingency awareness data for all possible genotype combinations are shown in Table 2.

There were no significant effects of *CRHR1* rs878886 genotype, *5-HTTLPR*/rs25531 genotype or their interaction with regard to age, STAI trait, NEO neuroticism, baseline startle magnitude, contingency awareness and the percentages null responses during the acquisition and expression phases (all *P*-values > 0.2; see Table 2 for descriptive data). The only genetic association with any of the measurements described above was between *CRHR1* rs878886 and intensity of the electrical shocks. During the shock workup, G-carriers rated the electrical shocks as less annoying/painful than C/C homozygotes. As shock intensities are adjusted so that each subject rates shocks at a level of 4 out of 5 at the end of the workup (representing 'quite annoying/painful'), G-carriers ended up with higher shock intensities than C/C homozygotes ($F_{1,204} = 4.53$, $P = 0.035$, $\eta^2 = 0.02$; see Table 2). Including shock intensity as a covariate in the statistical analyses that involve *CRHR1* rs878886 did not change the significance of the genetic associations reported in the following.

Startle results

Acquisition of cue conditioning

During the uninstructed acquisition phase, significant potentiation of the eye blink startle reflex to the threat cue was observed by contrasting startles during light on/CXT+ with startles during light off/CXT+ (cue FPS: $F_{1,204} = 28.4$, $P < 0.001$, $\eta^2 = 0.12$). Consistent with our initial findings, this potentiation toward the threat cue (cue FPS) was significantly modulated by *CRHR1* rs878886 genotype ($F_{1,199} = 6.42$, $P = 0.01$, $\eta^2 = 0.03$), as G-carriers showed a reduced cue FPS compared with C/C homozygotes. There was no main effect of *5-HTTLPR*/rs25531 genotype ($F_{1,199} = 1.41$, $P = 0.24$) on cue FPS. However, there was a significant interaction between both genetic polymorphisms ($F_{1,199} = 6.86$, $P = 0.01$, $\eta^2 = 0.03$), showing that the less pronounced cue-potentiation of the G-allele carriers was most prominent within the *5-HTTLPR*/rs25531 'high function' group (see Fig. 2a).

Acquisition of context conditioning

There was significant overall startle potentiation to the threat context as indexed by contrasting light off/CXT+ startles with light off/CXT− startles (context FPS: $F_{1,204} = 121.5$, $P < 0.001$, $\eta^2 = 0.37$). This contextual startle potentiation was significantly modulated by *CRHR1* rs878886 ($F_{1,199} = 4.67$, P = 0.03, $\eta^2 = 0.02$). G-allele carriers, which showed a decreased cued fear response as described above, demonstrated an increased context FPS compared with C/C homozygotes (see Fig. 2b). There was neither a *5-HTTLPR*/rs25531 main effect on context FPS ($F < 1$) nor was there a *CRHR1* × *5-HTTLPR*/rs25531 interaction effect ($F < 1$).

After the fear acquisition phase, all subjects were explicitly instructed about the shock contingency, both verbally and in writing (on-screen). Fear expression as measured by startle responses was then assessed in four more blocks. During this fear expression phase, there was significant potentiation of the startle reflex to the threat cue ($F_{1,204} = 290.6$, $P < 0.001$, $\eta^2 = 0.59$) and the threat context ($F_{1,204} = 159.7$, $P < 0.001$, $\eta^2 = 0.44$). Consistent with the initial study, no genetic modulations of startle activity were observed during this phase as both cue FPS and context FPS were independent of *CRHR1* rs878886 genotype, *5-HTTLPR*/rs25531 genotype and their interaction (all *P*-values > .12; see Fig. 2).

Subjective measures

Fear ratings

As in the initial study, there was significant potentiation of subjective fearfulness to both the threat cue and the threat context during uninstructed fear acquisition and instructed fear expression (all *P*-values < 0.001). None of the genetic factors under study were related to subjective fearfulness (all *P*-values > 0.28).

Reported awareness of shock contingency

According to the criteria for the processing of the forced choice data as described earlier (Baas 2013), 182 subjects (88.8%) were aware of the 'threat context – shock contingency' at the end of the acquisition phase, whereas 23 were unaware (11.2%). As in the initial study, all genetic factors under study were unrelated to awareness of the 'threat context – shock contingency' (all *P*-values > 0.37). With regard to the awareness of the 'light on – shock contingency' within the threat context, 79 subjects (38.5%) qualified as aware whereas 126 subjects (61.5%) qualified as unaware. This was a lower percentage of cue awareness than in our initial study (53.4% aware of the light on – shock contingency). However, this difference was not statistically significant ($\chi^2 = 2.88$, $P = 0.09$). Consistent with our initial findings, however, the genetic factors under study were again unrelated to cue awareness (all *P*-values > 0.065; see Table 1 for contingency awareness frequencies per genotype group). Furthermore, addition of cue awareness and context awareness as covariates for the statistical analyses involving genetic factors described in Startle results and Subjective measures did not change the genetic effects. This means, that all statistical test outcomes involving the genetic factors under study (significant or non-significant) were identical with and without inclusion of both these covariates.

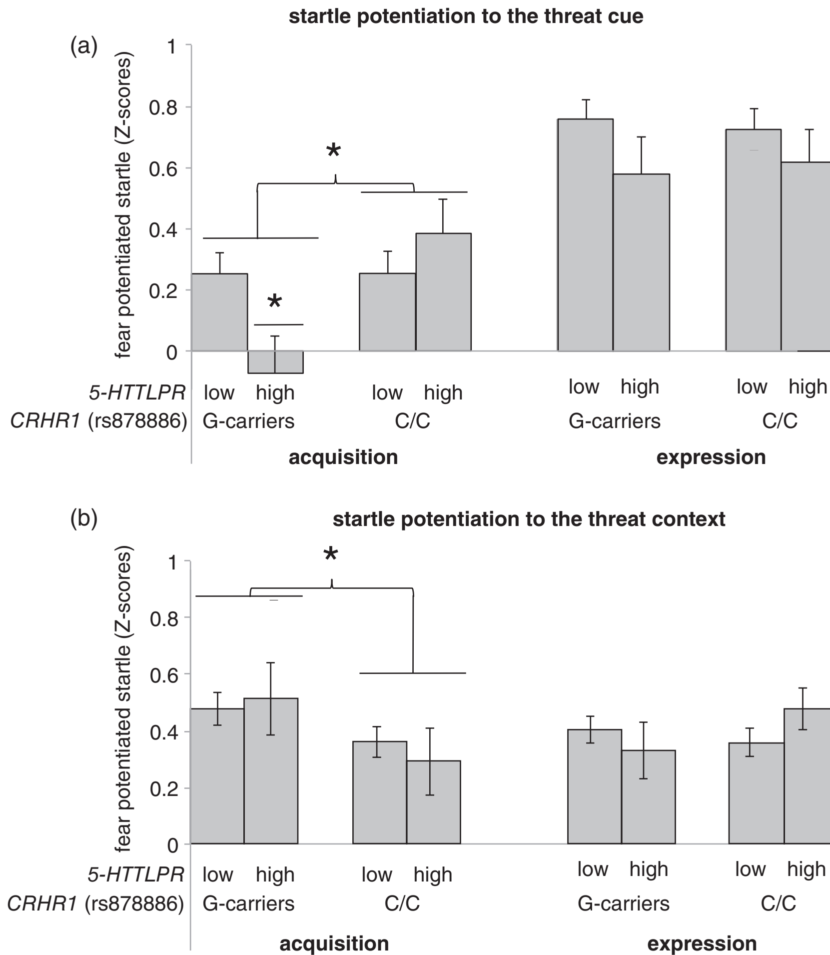

Figure 2: Potentiated-conditioned fear responses to the threat cue (panel a) and threat context (panel b) during the acquisition phase and the expression phase are plotted as a function of *CRHR1* rs878886 and *5-HTTLPR*/rs25531 genotypes. Significant effects denoted are the main effect of *CRHR1* rs878886 on cued fear responses during acquisition, defined as the contrast between light on/CXT+ vs. light off/CXT+, and an interaction effect of *CRHR1* x *5-HTTLPR*/rs25531 on cued fear during acquisition (both in panel a). In panel b, the significant effect marked with * is the main effect of *CRHR1* on contextual fear responses during acquisition, defined as the contrast between light off/CXT+ vs. light off/CXT−. *N*'s per genotype groups are *CRHR1* G-carriers, *5-HTTLPR* low functioning = 74; *CRHR1* G-carriers, *5-HTTLPR* high functioning = 23; *CRHR1* C/C, *5-HTTLPR* low functioning = 80; *CRHR1* C/C, *5-HTTLPR* high functioning = 28. Error bars display + 1 standard error of the mean; * $P = 0.05$.

Additional analyses on the aggregated sample

In addition to the line of analyses reported above, we computed statistics on the startle data of the aggregated sample including both the subjects of the original report (Heitland *et al.* 2013) and the subjects described here. As no triallelic genotyping was performed in the original study, we used the biallelic *5-HTTLPR* genotype as a factor for these analyses. The pattern of results in the replication sample was also observed in the analyses of the aggregated sample. As such, fear potentiation startle amplitudes toward the threat cue (cue FPS) were significantly modulated by *CRHR1* rs878886 genotype ($F_{1,345} = 9.52$, $P < 0.01$, $\eta^2 = 0.03$), as G-carriers showed a reduced cue FPS compared with C/C homozygotes. Furthermore, the significant modulation of context FPS by *CRHR1* rs878886 genotype was also present in the analyses of the aggregated sample ($F_{1,345} = 4.86$, $P = 0.03$, $\eta^2 = 0.01$). No significant modulations of cue FPS and context FPS were found in the expression phase, after explicit instructions were given, identical to what was observed in the replication sample (*P*-values > 0.17). Moreover, no significant modulations of 5-HTTLPR genotype on cue FPS or context FPS were observed (*P*-values > 0.35). As in the replication sample, there was a significant interaction between both genetic polymorphisms with regard to cue FPS ($F_{1,345} = 6.82$, $P < 0.01$, $\eta^2 = 0.02$).

Other genetic variants investigated in the current sample

Apart from the focus of this report, i.e. fear acquisition and genetic variability in *CRHR1* rs878886 and *5-HTTLPR*/rs25531, we have reported on other genetic polymorphisms in relation to fear expression (serotonin 1A receptor [*5-HTR1A* rs6295], see Baas & Heitland 2014) and fear extinction (cannabinoid receptor 1, [*CNR1* rs2180619], see Heitland *et al.* 2012) in the past using this paradigm. Given the availability of data from an identical paradigm in a novel sample, we conducted the same set of analyses as reported in Baas & Heitland (2014), in this sample. In contrast to the initially observed association between *5-HTR1A* (rs6295) and fear expression (as indexed by FPS), this association did not reach significance in this sample. Data pertaining to the association of genetic variability in the human *CNR1* (rs2180619) and fear extinction will be reported elsewhere (manuscript in preparation). Full data on both sets of analyses is available on request.

Discussion

Here, we conducted an exact replication study of Heitland *et al.* (2013) in a substantial sample ($N > 200$) to further substantiate the conclusions drawn initially. In the original study, we investigated the role of genetic variability within *CRHR1* and *5-HTTLPR*/rs25531 in relation to fear conditioning. Both neurotransmitter systems were selected based on earlier findings in both rodents and humans as discussed in the introduction. Fear potentiation of the eye blink startle reflex (FPS) was measured to assess both uninstructed fear acquisition and instructed fear expression within a well-established virtual reality fear-conditioning paradigm. Two effects were observed in the initial study. First, *CRHR1* G-allele carriers showed reduced acquisition of fear-conditioned responses (cue FPS) to the threat cue when compared with C/C homozygotes. Second, when *CRHR1* G-allele carriers were also carrying the *5-HTTLPR*/rs25531 less active alleles, this translated into heightened fear-conditioned responses to the threat context (context FPS).

In this replication study, *CRHR1* G-allele carriers again showed reduced potentiation of their fear-conditioned responses (cue FPS) compared with risk allele homozygotes. The main effect of *CRHR1* genotype on cued fear acquisition was thus observed across both the discovery and the replication sample. Moreover, in the replication sample, this deficit in cue acquisition present in G-allele carriers was accompanied by heightened contextual fear responses (context FPS). This pattern of results is consistent with the model that failure to condition to a specific predictor of threat leads to enhanced contextual anxiety (Baas *et al.* 2008; Baas & Heitland 2014; Grillon 2002; Heitland *et al.* 2013), and with the data by Keck *et al.* (2008) where G-allele carriers show an increased risk for panic disorder. After explicit instructions regarding the threat contingency were given, differences between genotype groups in fear-conditioned responses to both the threat cue and context disappeared, as observed in the initial study. This points toward fear acquisition specific effects of *CRHR1* gene variation. Furthermore, subjective fearfulness and contingency awareness were unrelated to all genetic factors of interest in both the replication and the initial study.

From this pattern of results, the first and foremost conclusion that can be drawn is that *CRHR1* rs878886 is associated with fear acquisition deficits in two independent, substantial samples of healthy human subjects ($N = 146$ and $N = 205$). In this new sample, we observe a direct replication of the initially observed main effect of *CRHR1* rs878886 on cued fear acquisition (Heitland *et al.* 2013). The current data fit with converging evidence from animal research (Bijlsma *et al.* 2011; Liang and Lee, 1988; Roozendaal *et al.* 2002) and, importantly, novel human data (Weber *et al.* 2015) that suggest CRH as a major factor in fear and anxiety across species. Of note, the *CRHR1* SNP associated with panic disorder and anxiety sensitivity in the latter human study (rs17689918) is in perfect linkage disequilibrium with the SNP under study here (rs878886). This means, that all subjects carrying a rs17689918 risk allele for the phenotype pertaining to increased fear sensitivity and heightened anxious apprehension as described in Weber *et al.* (2015), also carry the rs878886 risk allele for less cue acquisition and increased anxious apprehension toward contextual cues as shown here. Importantly, this implies that the functional implications of rs17689918 directly translate to rs878886 risk allele carriers. As both SNPs are perfect proxies to each other, the finding of less CRHR1 mRNA expression in the forebrain and amygdalae for the rs17689918 risk allele, regions crucial in the regulation of fear and anxiety, also directly apply to the risk (G) allele of rs878886. Accumulated evidence from the two earlier studies pertaining to human fear processing (Heitland *et al.* 2013; Weber *et al.* 2015) and this study thus allow for the conclusion that genetic variability in the human *CRHR1* gene leading to reduced CRHR1 expression is associated with a phenotype characterized by heightened sustained fear and deficient discriminative fear learning.

Mechanistically, our *CRHR1* findings, i.e. less cue acquisition and increased contextual anxiety for risk allele carriers, may provide novel insights into how CRHR1 might exerts its effects of fear regulation in humans. In traditional models, sustained (contextual) fear is mediated by CRH-driven activity of the BNST, whereas the CeA modulate acute, phasic fear responses (Davis *et al.* 2010), potentially via inhibitory CRH projections from the BNST to the CeA (Campeau *et al.* 1997; Haufler *et al.* 2013). In our study, CeA-driven phasic fear responding is measured by the cue FPS while the context FPS reflects BNST-driven sustained fear responses (Davis *et al.* 2010). This allows us to frame our findings in terms of the current models of CRH-effects on fear responding. Two alternative mechanisms are discussed below. Of note, psychophysiological recordings such as the FPS reported here can obviously not directly inform the underlying neurobiology, but other evidence suggests that the FPS is strongly related to amygdalar activity both in rodents (Davis *et al.* 2010), rhesus monkeys (Davis *et al.* 2008) and humans (Klumpers *et al.* 2010a), allowing for well-informed speculative models.

In one putative model, CRH-dependent underactivity of the CeA as suggested in risk allele carriers (G-allele of rs878886, A-allele of rs17689918) leads to reduced acquisition of phasic fear responses, thus less discriminative fear learning. Threat then remains unpredictable, as quick and adaptive up/down regulation when threat is present (light on in the shock context in this study) is not possible. As a consequence, subjects tend to be afraid regardless of whether the predictive cue is present or not, leading to chronic, sustained fear while in the environment where threat might occur. Such sustained anxious apprehension as a result of the failure to acquire cued fear has been demonstrated both experimentally (Baas *et al.* 2008; Baas & Heitland 2014) using the exact same paradigm as used here and clinically, e.g. in panic disorder patients (Wolpe & Rowan 1988). Notably, this model fits with recent rodent data (Bijlsma *et al.* 2011; Roozendaal *et al.* 2002) on CRF-dependent and amygdala-driven discriminative fear learning. Moreover, these animal data are translated by recent human data (Weber *et al.* 2015) as demonstrated by reduced discriminative activation of the amygdala during fMRI (CS+ vs. CS−) in risk allele carriers of rs17689918.

As a second possible model, CRHR1 might directly exert its effect on the BNST, leading to an overall tendency to display heightened anxious apprehension. This BNST effect might, in turn, hinder fear acquisition processes via its

inhibitory connection with the CeA (Campeau *et al.* 1997; Haufler *et al.* 2013), which itself is crucial in regulating acute, phasic fear responses. Of note, both models are not falsifiable by genetic association studies as performed here. Whether CRF-dependent effects on the CeA are associated with failure to acquire fear contingencies, leading to heightened contextual anxiety (model #1) or CRF-dependent effects on the BSNT lead to increased sustained fear, thereby hindering the acquisition of phasic fear responses (model #2) is an empirical question. Future mechanistic studies using, e.g. CRHR1 (ant)agonist administration combined with neuroimaging measurements during fear conditioning will allow more specific insights.

Apart from the replicated main effect of CRHR1 on fear acquisition, the data of both the discovery and the replication study also allow the conclusion that genetic variation in *CRHR1* interacts with *5-HTTLPR*/rs25531 regarding fear acquisition. In the original study, only *CRHR1* rs878886 G-allele carriers that carried at least one *5-HTTLPR*/rs25531 short allele showed heightened contextual fear. In this replication study, however, the heightened context responding was independent of *5-HTTLPR*/rs25531 genotype. Another difference between findings in the original versus the replication sample is that in the replication sample, there was a *CRHR1* rs878886 × *5-HTTLPR*/rs25531 interaction with regard to cue FPS (fear-conditioned responses to the threat cue during acquisition). *CRHR1* G-allele carriers with a *5-HTTLPR*/rs25531 'high functionality' profile, equivalent to L/L homozygotes in the initial study, showed considerably smaller startle responses to the threat cue than all other genotype groups. Note, that the 5-HTTLPR findings in the replication study are based on triallelic 5-HTTLPR analyses, whereas the initial study used biallelic genotype grouping. However, all triallelic effects pertaining to fear acquisition in the replication were also present when genotypes were grouped based on biallelic profiles. Whereas the interaction of *CRHR1* × *5-HTTLPR*/rs25531 was observed in relation to contextual fear acquisition in the discovery study, it was present on cued fear acquisition here. (Our) animal work, studying the 5-HTT × CRF1 receptor interaction on fear acquisition and contextual anxiety in rat potentiated startle, revealed yet another interaction (Bijlsma *et al.* 2015). These studies showed that reduced 5-HTT function during development (through knockout of 5-HTT gene) was associated with fear acquisition deficits and heightened contextual anxiety. Both could be normalized by acute treatment with a CRF1 receptor antagonist. This normalizing effect of the CRF1 receptor antagonist is line with previous work showing that subchronic central administration of CRF disrupted fear acquisition (Bijlsma *et al.* 2011). The various interactions of both neurotransmitter systems, however, make integrating and interpreting the current results into theoretical frameworks challenging, particularly as the molecular consequences of *CRHR1* rs878886 and its interaction with 5-HTT are not fully understood as of yet.

The notion that CRH and 5-HT interact, in particular with regard to anxiety, has received growing attention during the last years and more and more evidence is accumulating across species supporting their interplay (Hauger *et al.* 2009; Risbrough & Stein 2006). A multitude of potential underlying mechanisms for this interplay have been described (recently: Fox & Lowry 2013); see also (Homberg & Contet 2009; Reul & Holsboer 2002; Valentino *et al.* 2010) for reviews). The most pivotal neural structure where this interaction manifests is the dorsal raphe nucleus (dRN), where CRF regulates dRN-based serotonin activity and projections (Forster *et al.* 2008; Kirby *et al.* 2008; Valentino *et al.* 2010; Waselus *et al.* 2009). The interaction at the level of the dRN can be tied directly into the circuitry responsible for contextual fear responses, as CRF projections from the BNST activate serotonergic feedback projections from the raphe back to the BNST. This circuitry has been shown to downregulate the BNST and hence control levels of contextual fear (Hammack *et al.* 2003; Risbrough *et al.* 2009). However, this CRF-based regulation of serotonergic projections in the dRN depends on CRF receptor subtype, dose and other factors (Homberg & Contet 2009). This complex mechanism of action requires further study, especially when it comes to the mutual interactions between the two neurotransmitter systems and how this impacts cue and contextual fear learning.

What is becoming more and more apparent is that both direct and conceptual replication studies are essential to fully understand the complex roles that genetic variants can play in behavioral neuroscience studies (Simons 2014). Direct replication in a substantial sample of individuals as done in this study is relatively rare in the field of human behavioral genetics (Makel *et al.* 2012; Munafò & Gage 2013). Replication issues in psychological candidate gene association studies are very prominent, as the acquisition of one sufficiently large sample is already challenging, especially when using experimental designs and/or psychophysiological or neuroimaging measures. Even when multiple studies analyze the same genotypes in related phenomena, lack of replication may be due to a combination of several factors, which include small sample sizes leading to underpowered study designs, a posteriori instead of a priori selection of genetic targets, the measurements of processes rather distant from the biological function of the gene itself and the lack of validated standards to measure the neurobiological process of interest [see the following reviews and opinions for an overview on this topic (Bogdan *et al.* 2013; Button *et al.* 2013a, 2013b; Flint & Munafò 2013; Lonsdorf & Baas 2015; Meyer-Lindenberg 2012; Munafò & Gage 2013)]. We tried to account for these issues by measuring a substantial sample ($N > 350$ in the aggregated sample), selecting our target polymorphisms up front (determined in the protocol for ethics approval) and by choosing a genetic polymorphism that is linked to psychiatric prevalence (Keck *et al.* 2008), and neurophysiological measurements (Hsu *et al.* 2012). A posteriori validation comes from the recent demonstration of functional effects on *CRHR1* gene expression (Weber *et al.* 2015).

During the last decade, the above-mentioned issues have received a considerable amount of attention in the literature. As a result of this ongoing discussion, there is agreement that findings from candidate gene studies, especially when genetic interactions are considered, ought to be replicated before strong conclusions can be made. This is particularly the case for findings from neurotransmitter systems that have not been studied to a large extent yet. Fortunately, there have been a lot of recent developments aimed at facilitating

the publication of replication studies and raising awareness about their importance, such as the registered replication report initiative and many excellent guideline and opinion articles (Button *et al.* 2013a, 2013b; Flint & Munafò 2013; Ioannidis *et al.* 2014; Jasny *et al.* 2011; Munafò & Gage 2013; Simmons *et al.* 2011).

A limitation of this study is that even though the sample sizes are rather large in comparison with standards in the field, the current sample sizes of $N = 146$ and $N = 205$ still have limited power for the detection of genetic interaction effects as a minimum of four groups have to be used in statistical analyses. Therefore, definitive conclusions with regard to the *CRHR1* × *5-HTTLPR* interaction on fear acquisition must await further examination.

Taken together, we here provide a replication of the earlier reported association of genetic variability in the human *CRHR1* gene and fear acquisition (Heitland *et al.* 2013). Together with recent animal and human data, our findings suggest potential mechanistic pathways through which CRHR1 may play an important role in the pathogenesis of anxiety disorders.

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